Increased survival and reduced renal injury in MRL/lpr mice treated with a novel sphingosine-1-phosphate receptor agonist

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Agonists of the type 1 sphingosine-1-phosphate (S1P) receptor inhibit lymphocyte migration, causing their sequestration in lymphoid tissue. The S1P agonist FTY720 prolongs the survival of organ allografts and blocks T-cell mediated autoimmune diseases in experimental models; however, it is a non-selective agonist of four of the five S1P receptors. In this study female MRL/lpr mice, which develop an aggressive form of spontaneous autoimmune kidney disease, were treated with a more selective agonist of the type 1 receptor (KRP-203) or vehicle at 12 or 16 weeks of age. Eighty percent of the mice treated at 12 weeks, before the onset of visible disease, survived to the 24 weeks end point with decreased tubulointerstitial disease and significantly fewer infiltrating $CD4^+$ and $CD8^+$ T-cells. Only half of the control vehicle-treated mice survived. All of the mice treated at 16 weeks survived with reduced proteinuria. Mice in both groups had significant reductions in circulating lymphocytes. Mice receiving KRP-203 for 8–12 weeks had significant reductions in T-cells and consequently less adenopathy. *Ex vivo* treatment of lymphocytes from MRL/lpr mice with KRP-203 enhanced their apoptosis. Our study indicates that KRP-203 attenuates kidney injury in MRL/lpr mice, in part, by reducing T-cell infiltrates.

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Systemic lupus erythematosus is a chronic autoimmune disease that causes tissue injury in a T-cell-dependent manner. Lupus nephritis is a leading cause of morbidity and mortality in systemic lupus erythematosus, and is characterized by proliferative glomerulonephritis, tubulointerstitial disease, and renal vasculitis. The homing of activated T cells to the kidney in lupus nephritis is thought to occur in response to deposition and local production of immune complexes with complement activation and generation of other proinflammatory mediators.[1,2] Current therapeutic agents, primarily steroids and cyclophosphamide, target T cells via nonspecific inhibition of activation or proliferation. Their use, however, has been associated with significant morbidity.[3,4] An alternative approach is to block recruitment and prevent accumulation of lymphocytes within local sites of inflammation.

Agonists of the type 1 sphingosine-1-phosphate ($S1P_1$) receptor are important new immunosuppressive agents.[5] The $S1P_1$ receptor is present on lymphocytes[6,7] and controls their migration throughout the body.[8,9] S1P is present in blood and mediates egress of lymphocytes out of secondary lymphoid organs back into the circulation.[9,10] FTY720 was the first S1P receptor agonist developed,[11] and its administration resulted in sequestration of peripheral lymphocytes into secondary lymphoid organs reducing lymphocytic infiltrates in target organs. FYT720 use prolongs allograft survival and attenuates autoimmune-mediated injury in both humans and experimental animals.[5,12–16]

Unfortunately, FTY720 has been associated with unanticipated side effects in clinical trials, principally bradycardia, renal fibrosis, and macular edema. These side effects have been attributed to the nonselective S1P receptor activity of FTY720.[17–19] S1P receptors are a family of G-protein-coupled receptors. FTY720 is a potent agonist of four out of the five known S1P receptors, and the $S1P_3$ receptor has been shown to mediate many of these side effects.[17–19] In addition, FTY720 has been shown to activate the cardiac G-protein-gated potassium channels resulting in bradycardia *in vivo*.[20] Lymphocyte egress from lymph nodes is mediated primarily by the $S1P_1$ receptor.[9,18,19] Therefore, there is reason to

believe that selective $S1P_1$ receptor agonists would be effective immunomodulators without the side effects noted with FYT720.

KRP-203 (2-amino-2-(2-(4-(3-benzyloxyphenylthio)-2-chlorophenyl)ethyl)-1,3-propanediol hydrochloride) is a 480 Da compound with a molecular structure similar to FTY720. KRP-203 is selective for $S1P_1$ and $S1P_4$ receptor subtypes, with no effect on $S1P_3$ receptors.[21] KRP-203 accelerates lymphocyte homing into secondary lymphoid organs and blocks their egress similar to FTY720.[16,21,22] In rodents, KRP-203 has been effective in preventing rejection of skin and heart transplants, with reduced lymphocyte infiltration into these grafts.[22,23] In addition, it prevented autoimmune myocarditis in rats[24] and inflammatory liver disease and colitis in mice.[16,21] Importantly, the cardiac and vision effects seen with FY720 have not been described with KRP-203.[22]

In the present study, KRP-203 was tested for efficacy in a lymphocyte-dependent model of autoimmune nephritis. Autoimmunity and kidney disease occur spontaneously in mice homozygous for the lpr mutation in the MRL genetic background (MRL/lpr). In MRL/lpr mice, lymphocytes accumulate in the kidney where they mediate tissue damage. We postulated that KRP-203 treatment would result in sequestering of pathogenic lymphocytes in lymphoid tissues and away from target organs, thereby decreasing kidney injury and improving survival.

RESULTS

MRL mice that received vehicle only had 50% mortality by 24 weeks of age (Figure 1). However, mice in the treatment group showed no mortality by 24 weeks ($P = 0.02$). Although there was a clear trend toward improved survival in the prevention group, this did not reach statistical significance. This was primarily due to the loss of two mice that died early in treatment; importantly, there were no further deaths in this group either as the mice aged. Dermatitis was variably present, and there was a modest improvement in groups treated with KRP-203. Mice in all groups had equivalent weight gains throughout the study.

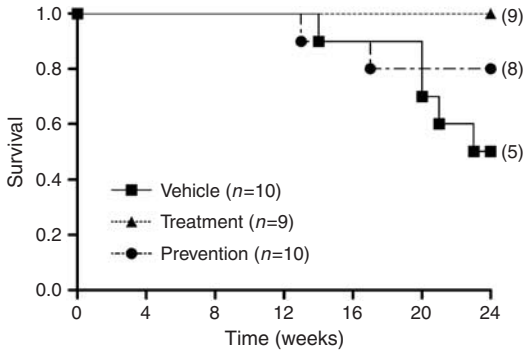

Figure 1 | KRP-203 Prolongs survival of MRL/lpr mice. Kaplan–Meyer curves showing survival of mice treated with vehicle alone (squares) or KRP-203 6 mg/kg per dose three times per week starting at either 12 weeks (circles) or 16 weeks of age (triangles). Numbers of mice surviving to 24 weeks are shown in parentheses. *P*-value (treatment vs vehicle) = 0.02; *P*-value (prevention vs vehicle) > 0.05.

Histologic analysis

As inflammatory kidney disease is the primary cause of death in MRL/lpr mice, renal histology was assessed at 24 weeks. As expected, kidneys from control mice had severe proliferative glomerulonephritis, chronic tubulointerstitial disease, and large perivascular infiltrates (Figure 2a, d and g). Consistent with the model, most glomeruli were hypercellular with obliteration of capillary loops, focal segmental sclerosis, and occasional fibrocellular crescents. Immunofluorescent antibody staining for C3 and immunoglobulin G (IgG) showed the typical pattern with diffuse global staining of mesangium and capillary loops. There was interstitial fibrosis with diffuse infiltration of mononuclear cells, tubulitis, and focal tubular atrophy. Large and small vessels were also surrounded by large mononuclear cell infiltrates. Immunofluorescence staining showed that mononuclear infiltrates consisted mostly of $CD4^+$ or $CD8^+$ T cells, with fewer $CD11b^+$ monocytes/neutrophils and $CD138^+$ plasma cells (data not shown).

Mice in the prevention group had reduced histopathology scores. There were lower glomerular cellularity scores ($P = 0.06$), and, unlike vehicle-treated controls, none of the sections analyzed contained glomerular crescents (Table 1). There was no difference in the degree or distribution of complement or immunoglobulin staining. However, there was significantly less tubulointerstitial disease (Table 2) with preservation of tubular architecture (Figure 2e). Although there was an overall trend toward less vasculitis ($P = 0.29$), there was substantial intragroup variability between mice. There was an eightfold reduction in periglomerular $CD4^+$ and $CD8^+$ T cells and there were also fewer T cells in the interstitium. $CD11b^+$ and $CD138^+$ cells were present in numbers equivalent to kidneys from control mice (data not shown).

Mice in the treatment group had intermediate histopathology scores. Although there was a trend toward decreases in glomerular cellularity, the proportion of glomeruli with crescents, tubulointerstitial disease and the size of perivascular infiltrates (Tables 1 and 2), none of these differences achieved statistical significance. Immunofluorescence revealed no difference in complement or immunoglobulin staining, but there were 50% fewer $CD4^+$ and $CD8^+$ T cells around glomeruli and within the interstitium.

Renal function

Urine protein excretion increased in all mice from 8 to 16 weeks of age. In the treatment group, therapy was associated with a significant reduction in proteinuria at 20 weeks of age compared to controls (0.36 ± 0.03 mg/kg/day vs 0.55 ± 0.07 mg/kg/day, respectively; $P < 0.05$). In the prevention group, half the mice had a marked reduction in urinary protein excretion, but others did not (mean 0.42 ± 0.09 mg/kg/day).

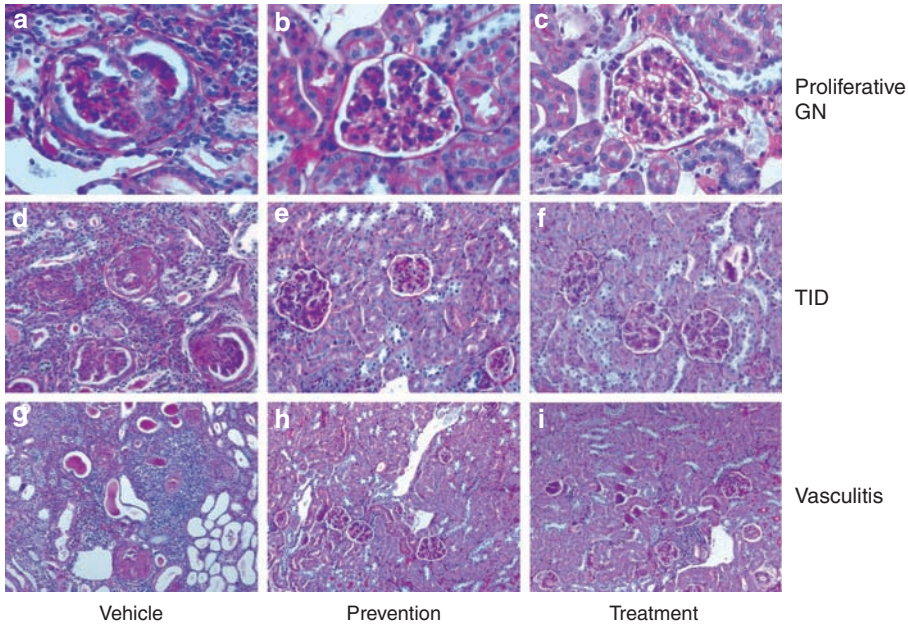

Figure 2 | Mice treated with KRP-203 have less inflammatory glomerulonephritis and reduced interstitial and perivascular infiltrates. Representative micrographs of 4-μm PAS-stained sections from paraffin-embedded tissue harvested from mice at 24 weeks of age. (**a**–**c**) Original magnification ×400. Glomeruli from mice treated with vehicle alone had more sclerosis, crescents, and periglomerular infiltrates (**a**), whereas glomeruli from mice in the prevention (**b**) or treatment (**c**) groups had less proliferative lesions with fewer periglomerular cells. (**d**–**f**) Original magnification ×200. Cortex from mice treated with vehicle only had large inflammatory infiltrates with tubular atrophy and loss of normal architecture (**d**). Cortex from mice in the prevention (**e**) and treatment (**f**) groups had more preserved architecture and reduction in interstitial infiltrates. (**g**–**i**) Original magnification ×100. Bridging renal vessels from mice treated with vehicle alone had large circumferential infiltrates that extended into the cortical interstitium (**g**), whereas equivalent vessels from mice in the prevention (**e**) and treatment (**f**) groups had smaller and irregularly distributed infiltrates.

Table 1 | Glomerular histology scores

	Vehicle (n=6)	Prevention (n=8)	Treatment (n=9)
Hypercellularity	2.25 ± 0.19	1.00 ± 0.29*	1.80 ± 0.15
Crescents	13 ± 9.4%	0 ± 0%	8.3 ± 6.8%
C3 staining	1.85 ± 0.16	1.94 ± 0.20	2.08 ± 0.17
IgG staining	1.15 ± 0.13	1.07 ± 0.06	1.15 ± 0.17
$CD4^+$ cells/glomerulus	12.2 ± 3.6	1.5 ± 0.3**	6.8 ± 1.1
$CD8^+$ cells/glomerulus	5.7 ± 1.5	0.8 ± 0.1**	3.8 ± 1.1

IgG, immunoglobulin.
*P-value (prevention vs vehicle) <0.05;
**P-value <0.01.

Table 2 | Interstitial histology scores

	Vehicle (n=6)	Prevention (n=8)	Treatment (n=9)
Vasculitis	3.0 ± 0.00	1.67 ± 0.54	1.75 ± 0.59
Tubulointerstitial disease	3.0 ± 0.00	0.5 ± 0.20*	0.8 ± 0.15
$CD4^+$ cells/hpf	21.3 ± 4.0	6.6 ± 0.8*	11.6 ± 1.3
$CD8^+$ cells/hpf	20.1 ± 1.5	4.7 ± 0.9*	9.5 ± 2.3†

hpf, high-powered field.
*P-value (prevention vs vehicle) <0.05,
†P-value (treatment vs vehicle) <0.05.

Serum creatinine measurements at 24 weeks showed equivalent levels in all three treatment groups: control 0.34 mg/100 ml ± 0.02, prevention 0.38 mg/100 ml ± 0.02, and treatment 0.40 mg/100 ml ± 0.07.

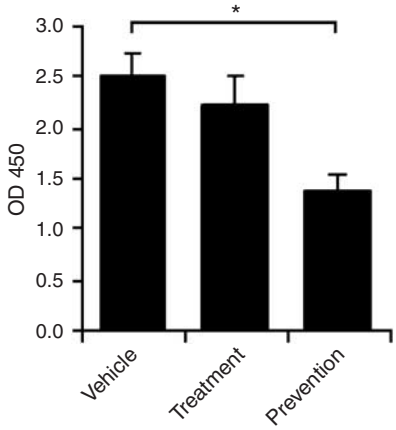

Figure 3 | Duration of treatment-dependent decrease in anti-double-stranded DNA titers. Absorbance at 450 nm shown from ELISA using 1:400 dilutions of serum from mice treated with vehicle alone or KRP-203 for 8 or 12 weeks. Values represent the means of 8–10 mice per group and bars reflect the standard error. *P-value <0.05.

Autoantibodies

All mice had elevated anti-double-stranded DNA antibody titers compared to pooled serum from nonautoimmune mice. There was a modest reduction in anti-double-stranded DNA antibody titers in treated mice and the effect seemed to correlate with duration of therapy (Figure 3). This

corresponded to less than a fourfold reduction in end point titers. There were no detectable differences in isotype-specific titers (data not shown).

Leukocyte analysis

At 24 weeks, there was a significant decrease in both peripheral lymphocytes (sixfold) and monocytes (fivefold) in both the prevention and treatment groups compared to controls (Figure 4). There were no significant differences in other leukocyte subsets or in the level of anemia. Mice in all three groups had equivalent platelet counts in the low normal range (0.3–0.7 million per μl).

All mice had subjective adenopathy at 12 weeks of age, and this was observed to increase in mice from all three groups up to 16 weeks. After 8–12 weeks of therapy in the treatment and prevention groups, respectively, there were decreases in lymph node size and splenocyte weight compared to control mice (Figure 5). At 24 weeks, there were 70% fewer lymph node cells in mice from the treatment group. This was due to reductions in all T-cell subsets (Table 3), but the greatest difference was the fourfold decrease in the $CD4^-CD8^-$ T-cell population in the lymph nodes ($P<0.0001$). The effect of KRP-203 therapy on reduced lymphocyte numbers was even more pronounced in mice from the prevention group. There were fivefold decreases in $CD4^+$ and $CD8^+$ T cells and 12-fold decreases in $CD4^-CD8^-$ T cells in the lymph nodes of mice from the prevention group. There was no difference in the numbers of $CD4^+CD25^+$ T cells between groups. There were similar reductions in B cells and neutrophils in the lymph nodes of mice in the prevention and treatment groups, but no difference in a numbers of $CD11b^+$ cells (data not shown). Similar findings were found in the spleen.

To assess potential mechanisms for the reduced lymphadenopathy seen in MRL/lpr mice treated with KRP-203, lymphocyte survival in *ex vivo* cultures was assessed after treatment with increasing doses of KRP-203 or vehicle control. Lymphocytes were isolated from axillary lymph nodes of young female MRL/lpr mice at 10 weeks of age. Nodes are moderately enlarged in mice at this age, and 90% of lymph node cells are T cells with 5–10% $CD4^+$, 5–10%

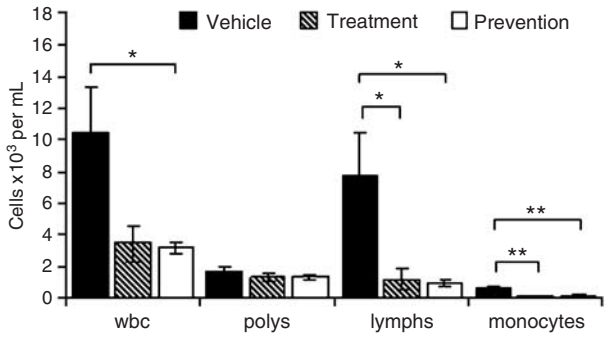

Figure 4 | Mice treated with KRP-203 have reduced numbers of circulating leukocytes. Absolute numbers of total white blood cells (wbc), polymorphonuclear cells (polys), lymphocytes (lymphs), and monocytes were measured from EDTA-treated whole blood at 24 weeks. Values are means of samples from five mice per group and bars reflect the standard error. *P-value <0.05; **P-value $\leqslant 0.005$.

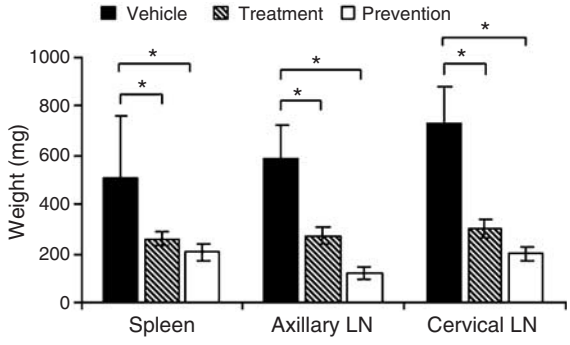

Figure 5 | KRP-203 reduces splenomegaly and lymphadenopathy in MRL/lpr mice. Spleen and lymph node (LN) weights measured at time of harvesting, at 24 weeks or at time of death. Values are means of samples from 9 to 10 mice and bars reflect standard error. *P-value <0.05.

Table 3 | Lymphocyte numbers in lymph node

	Vehicle (n=5)	Prevention (n=8)	Treatment (n=9)
LN $CD4^+$	29.2 ± 8.5	6.3 ± 2.3*	17.8 ± 6.5
LN $CD8^+$	8.2 ± 2.0	2.5 ± 0.9**	5.1 ± 2.1
LN $CD4^-CD8^-$	130.3 ± 16.9	10.8 ± 4.5***	30.0 ± 4.4†

LN, lymph node.
Absolute number of cells per node ($\times 10^6$).
*P-value <0.001;
**P-value (prevention vs vehicle) <0.05;
***P-value <0.0001;
$^\dagger P$-value <0.0001.

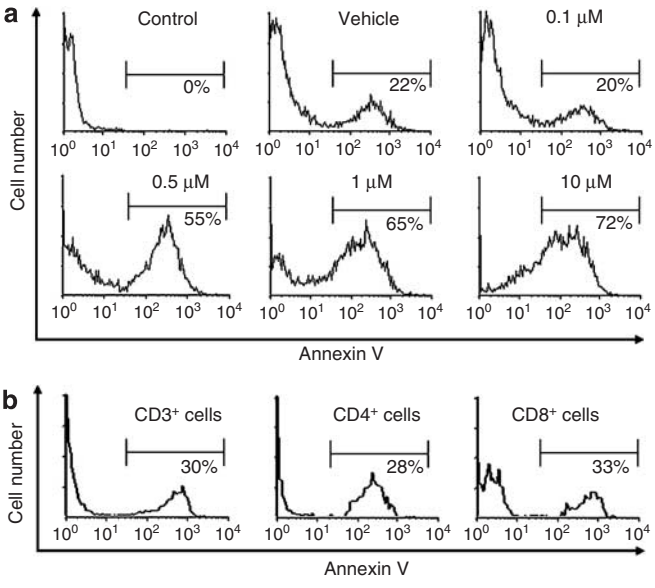

Figure 6 | KRP-203 enhances apoptosis of lymphocytes from MRL/lpr mice. (**a**) Histograms of cells stained for Annexin V-FITC after 24 h incubation with vehicle or increasing concentrations of KRP-203. Histogram of unstained cells included as a negative control. Markers indicate positive cells and percent positive cells are shown in each figure. Data are representative of three separate experiments. (**b**) Annexin V-FITC staining of T-cell subsets after gating on either $CD3^+$, $CD4^+$, or $CD8^+$ populations. Lymph node cells were treated with 0.5 μm KRP-203 for 8 h before staining.

$CD8^+$, and 70–80% $CD4^-CD8^-$ cells. After 24 h unstimulated in media with serum, 80% of cells were viable by Trypan blue exclusion or propidium iodide staining. Apoptosis was assessed by flow cytometry after immunostaining with Annexin V (Figure 6a). Treatment with KRP-203 revealed a dose-dependent increase in the proportion of cells staining positive for Annexin V. Using 10 mM KRP-203, a time course experiment revealed that 50% of cells were Annexin V positive at 4 h and nearly all cells were positive by 8 h. Only 20% of untreated cells or cells treated with dimethyl sulfoxide were Annexin V positive at 8 or 24 h. To determine whether there were any differences in KRP-203-induced apoptosis in specific T-cell populations, lymph node cells were exposed to drug for 8 h. The percentage of Annexin V-positive cells was similar in both the $CD4^+$ and $CD8^+$ subsets as compared to the entire $CD3^+$ population (Figure 6b).

DISCUSSION

The present study shows that KRP-203 is effective in ameliorating chronic inflammatory nephritis in the MRL/lpr mouse. An 8-week course of therapy starting after the mice show signs of systemic disease and renal involvement resulted in less proteinuria, reduced $CD8^+$ cell infiltrates, and prolonged survival. Further benefit was seen with a longer duration of therapy starting earlier in the course of disease. Mice treated for 12 weeks had even fewer circulating lymphocytes, reduced $CD4^+$ and $CD8^+$ cell infiltrates in the kidney, and less tubulointerstitial disease.

These results are consistent with prior reports on the use of FTY720 in the MRL/lpr model.[25,26] Daily oral dosing with FTY720 at 1 mg/kg per dose starting at 16 weeks of age and lasting 2 weeks led to prolonged survival, although renal function and kidney histopathology were not assessed. In total, 9 of 10 treated mice survived 38 weeks, compared to only 50% survival in untreated mice.[25] In another study, oral dosing with 2 mg/kg per dose three times weekly starting at 16 weeks of age and continuing until 10 month of age was compared to vehicle alone. Mice receiving FTY720 had prolonged survival, reduced anti-double-stranded DNA antibody titers, and reduced IgG staining in glomeruli of kidneys.[26]

This study adds to the evidence that S1P receptor agonists can inhibit disease in MRL/lpr mice. There were reduced lymphocyte infiltrates in the kidneys of KRP-203-treated mice: 8 weeks of therapy resulted in fewer $CD8^+$ T cells in the kidney, and 12 weeks of therapy resulted in reductions in both $CD4^+$ and $CD8^+$ T cells, suggesting that this effect was dependent on the duration of therapy. In addition, the decreased inflammation in the prevention group was associated with reduced tubulointerstitial disease. Similar to Okazaki *et al.*,[26] KRP-203 therapy led to reductions in anti-double-stranded DNA antibody titers. Unlike Okazaki *et al.*, we found no differences in glomerular staining for IgG or complement. However, the reduced T-cell response in KRP-203-treated mice was associated with reduced proteinuria even without affecting the degree of IgG or C3 deposition.

Baseline body weights, proteinuria measurements, and gross examination for adenopathy immediately after randomization revealed no differences between the groups. Twenty-four weeks was chosen as the time point for censuring the survival analysis, due to an expected 50% mortality in control group. As early mortality in MRL/lpr mice is primarily due to renal failure,[2] death of the sickest mice in the control group may have reduced the power to detect differences in serum creatinine and glomerular injury scores. Despite this, statistically significant differences in several measurements of disease severity were identified.

One important aspect of this study is the long duration of therapy, as opposed to the short courses described in most previous studies of KRP-203. Studies in rats looking at cardiovascular, hepatic, and renal effects of FTY720 used doses up to 5 mg/kg/day for 21 days.[27] With activation by FTY720, S1P receptors are internalized and degraded, thereby resulting in a cell that loses responsiveness to gradients of S1P expression for chemotaxis.[28] Initial agonist effects can be mediated by G-protein activation, but with receptor downregulation these effects should be short lived. Therefore, the effects of long-term therapy with S1P agonists are likely to be the result of a loss of S1P receptor signaling. It is not known whether KRP-203 also downregulates $S1P_1$ after binding. However, this study showed that there were no obvious effects on growth or vitality after a 3-month course of therapy.

The long-term exposure to KRP-203 was associated with lymphopenia as well as reduced lymphocyte numbers in the spleen and lymph nodes. Lymphopenia has been seen in other studies with S1P agonists and is expected due to their effect on blocking egress from lymphoid organs. However, despite initial progressive adenopathy with KRP-203, there was ultimately a reduction in spleen and lymph node weights as well as a decrease in the absolute number of lymphocytes in these organs. The reduction was dependent on the duration of therapy, and involved all T-cell subsets, including $CD4^-CD8^-$ T cells, which specifically survive in the MRL/lpr mice because of their intrinsic defect in Fas-mediated apoptosis.[29] The mechanism responsible for lymphocyte depletion with long-term therapy is unclear, although enhanced apoptosis via a Fas-independent pathway is a likely possibility.

An apoptotic effect of S1p agonists on MRL/lpr lymphocytes has been described previously.[25,26] FTY720 has been reported to promote apoptosis *in vitro*, although the occurrence of this effect *in vivo* has been greatly debated.[5] Both KRP-203 and FTY720 are derivatives of sphingosine, a metabolite in phospholipid metabolism. Sphingosine and its parent molecule ceramide have both been found to promote apoptosis via intracellular signaling.[30,31] FTY720 requires phosphorylation *in vivo* for effect, and the responsible enzyme is sphingosine kinase type 2.[32] It is unclear if FTY720 or phosphorylated FTY720 serves as a substrate for other enzymes such as S1P phosphorylase or ceramide synthase that act on S1P and sphingosine, respectively, to

generate ceramide. KRP-203 also requires phosphorylation to function,[23] and it is assumed that this occurs *in vivo* by type 2 sphingosine kinase. Potential metabolites of FTY720 and KRP-203 could act directly as a second messenger to promote apoptosis, or alternatively they could indirectly act by modulating intracellular levels of sphingolipids. Furthermore, sphingosine and ceramide act downstream of Fas signaling,[30] and so T cells from Fas-deficient MRL/lpr mice could be especially sensitive to S1P agonist induced apoptosis. The largest decrease in peripheral T cells was within the $CD4^-$ $CD8^-$ cell subset; although receptor expression on this cell population has not been confirmed, *in vitro* KRP-203 appeared to effect all T-cell populations uniformly.

Alternatively, lymphocytes may die of neglect in the absence of persistent antigenic exposure. Previous studies have shown that FTY720 treatment results in the accumulation of macrophages in lymph nodes.[33] Although reduced circulating monocytes were seen in KRP-203-treated mice, monocyte numbers within lymphoid tissue were not altered. It is unclear from the present study whether these monocytes are activated or not. If the antigen-presenting cells in the lymphoid tissue of KPR-203 mice are not sufficiently activated, T cells sequestered in nodes may be deprived of requisite costimulatory signals and thus have a heightened susceptibility to apoptotic death. However, apoptosis would have to occur in a Fas-independent manner.

Other potential explanations for lymphocyte depletion in lymphoid organs of KRP-203-treated mice seem less likely. Although polymorphonuclear cell infiltration of lymphoid organs has been reported in MRL/lpr mice treated with a 2 weeks course of FTY720,[25] lymph nodes from mice after long-term KRP-203 therapy were not grossly necrotic, and these nodes contained fewer polys than nodes from control animals. There was no evidence of lymphocyte egress from lymphoid tissues back into the blood to explain the decreased adenopathy. Therefore, apoptosis seems to be the most likely mechanism.

Due to the widespread distribution of S1P receptors, multiple cell types have been shown to respond to S1P agonists. In addition to effects on lymphocytes, drug effects on other cells could contribute to attenuation of disease in lupus or lupus-like diseases. In the current study, therapy with KRP-203 resulted in decreases in blood monocytes as well as lymphocytes. This could be an indirect effect due to changes in T-cell activation, or more likely a direct effect of KRP-203 on monocytes. Receptor expression has been detected on human monocytes and mouse macrophages, and exogenous S1P also mediates activation and chemotaxis of monocytes/macrophages.[33,34] In the kidney, rat mesangial cells express $S1P_2$, $S1P_3$, and to a lesser extent $S1P_1$ and $S1P_5$.[35,36] S1P binding to $S1P_2$ and $S1P_3$ are required for platelet-derived growth factor-induced mesangial cell proliferation.[36] Treatment of mesangial cells with S1P agonists *in vitro* upregulates the activation of SMADs and the expression of collagen IV, CTGF, and TIM1.[17] Although typically this would lead to increased fibrosis, use of S1P agonists in an *in vivo* glomerulosclerosis model showed attenuation of disease.[37] Peters *et al.* treated animals after inducing a form of glomerular injury where lymphocyte infiltration is not typically seen, suggesting that attenuation of disease was due to loss of S1P signaling in mesangial cells, likely from receptor downregulation. Although renal endothelial cells have not been examined, other types of vascular endothelial cells have been reported to express $S1P_1$, $S1P_2$, and $S1P_3$.[38] Endothelial cells respond to S1P agonists with colocalization of adhesion molecules in tight junctions.[33] These proteins control leukocyte migration across the endothelial barrier,[12] perhaps contributing to reduced homing of lymphocytes from the blood into local sites of inflammation. The receptor subtypes responsible for these cellular responses are being elucidated, but the selectivity of KRP-203 should minimize nonlymphocyte effects.

In conclusion, treatment with KRP-203 improved survival and reduced proteinuria in MRL/lpr mice. Earlier, more prolonged therapy was associated with reduced tubulointerstitial disease and fewer T-cell infiltrates in the kidneys of these mice. There is also a marked reduction in adenopathy and T-cell numbers in the spleen and lymph node, likely due to induction of Fas-independent apoptosis. This study supports the hypothesis that selective $S1P_1$ agonists may benefit patients with lupus nephritis and other chronic cell-mediated autoimmune diseases.

MATERIALS AND METHODS

Mice

MRL/MpJ-Tnfrsf6^{lpr} mice (Jackson Laboratories, Bar Harbor, ME, USA) were maintained in our animal colony on standard mouse chow. Only female mice were used in this study, as the disease phenotype is more severe in females. The study was approved by the University of Texas Health Science Center Animal Welfare Committee.

Experimental design

KRP-203 was obtained from Kyorin Pharmaceutical Co. Ltd (Tokyo, Japan) and reconstituted in 0.5% methylcellulose (Sigma, St Louis, MO, USA) at 0.8 mg/ml. Methylcellulose was used as the vehicle control. Female mice were randomly assigned to one of three groups at 12 weeks of age. Mice in the prevention group received KRP-203 at 6 mg/kg per dose three times per week by gavage from 12 weeks of age until 24 weeks. Mice in the treatment group were dosed similarly but starting at 16 weeks of age. Mice in the control group received vehicle only by gavage three times per week starting at 12 weeks of age. Mice were monitored daily for signs of drug toxicity or disease exacerbations. Weights were obtained twice monthly and drug dosages were adjusted accordingly.

Histologic analysis

Kidney tissue was fixed in phosphate-buffered saline 4% formalin, dehydrated and embedded in paraffin. Sections (4 μm) were stained with periodic acid Schiff. Glomerular injury was graded in a blinded manner as follows, with a minimum of 20 glomeruli scored per animal: percentage of glomeruli containing cellular crescents, percentage of glomeruli with sclerosis involving >25% of the glomerular tuft, and degree of hypercellularity (0–3 scale).

Tubulointerstitial disease was graded on a 0–3 scale as follows, with 10 high-power fields (HPF) scored per animal: 0, no cellular infiltrates with back to back tubules, no evidence of fibrosis; 1, 0 to 5 cells per HPF and minimal fibrosis; 2,5 to 10 cells per HPF with moderate fibrosis; and 3, >10 cells per HPF with marked fibrosis.

Immunostaining

OCT-embedded snap-frozen 4 μm sections of kidneys were used for direct staining with C3 (MP Biomedicals, Solon, OH, USA) and IgG-specific antibodies (Zymed, South San Francisco, CA, USA). Control staining was also performed using matched isotypes or IgG (data not shown). Staining was scored on a relative scale of 0–3 by two blinded reviewers for at least 10 glomeruli per mouse. Two-step indirect staining was performed for CD8 and CD4 using rat primary antibodies 53–6.7 and RM4-5, respectively, (BD Pharmigen, San Jose, CA, USA) with a FITC-conjugated donkey anti-rat secondary antibody (Jackson Immunoresearch, West Grove, PA, USA). Quantitation of staining was graded based on the number of positive cells per glomerulus or per HPF for tubular infiltrates, with a minimum of 10 glomeruli or HPF per mouse examined.

Renal function

At 24 h, urine collections were obtained from mice every 4 weeks and immediately before histologic analysis. Urinary protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA) and normalized for body weight. Blood was obtained by cardiac puncture and serum was collected after allowing blood to clot at 37 °C for 1 h. Serum creatinine was determined using HPLC.[39]

Leukocyte analysis

Blood was obtained by cardiac puncture at time of histologic analysis and placed in tubes containing EDTA. Complete blood cell counts were performed at MD Anderson Cancer Center veterinary core facility. Splenocytes and axillary lymph node cells were prepared from mechanical dissociation through 70-μm filters. Erythrocytes were eliminated via hypo-osmolar lysis. Leukocytes were washed and stained using directly conjugated antibodies and assessed by flow cytometry using the FACS Caliber (BD Biosciences). Antibodies were obtained from eBiosciences: rat anti-mouse CD4 (clone GK1.5), rat anti-mouse CD8 (53–6.7), hamster anti-mouse CD3 (145-2c11), rat anti-mouse CD11b (M1/70), rat anti-mouse B220 (RA3-6B2), and rat anti-mouse CD138 (281-2).

Measurement of antibodies

Serum levels of antibodies reactive to double-stranded DNA were measured by enzyme-linked immunosorbent assay, as described previously.[40]

Apoptosis assay

Lymphocytes were isolated from axillary lymph nodes of young 10- to 12-week-old female MRL/lpr mice by mechanical dissociation and washing in sterile Hank's balanced salt solution. Cells (1×10^6) were plated in 1 ml complete RPMI medium with 10% fetal bovine serum in 24-well tissue culture dishes and incubated at 37 °C and 5% CO_2. A stock solution of 10 mM KRP-203 was made in dimethyl sulfoxide and dilutions were made into RPMI. Dimethyl sulfoxide at the appropriate dilution was used as a negative control. Annexin V staining was performed using the apoptosis detection kit (BD Pharmingen) and cells were analyzed by flow cytometry. Histograms were generated using WinMDI software, version 2.9 (Scripps, San Diego, CA, USA). For T-cell subset analysis, two-color staining was performed using antibodies for CD3, CD4, or CD8 (BD Pharmingen).

Statistics

Analyses performed using Microsoft Excel and GraphPad Prism software. Survival data were tested by Kaplan–Meyer analysis with significance determined by the log-rank test. Kruskal–Wallis test was used to compare other parameters.

DISCLOSURE

All the authors declared no competing interests.

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